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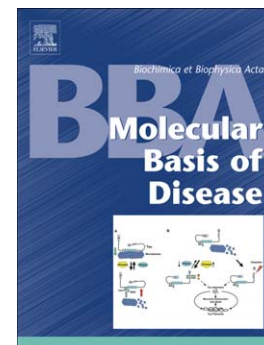
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The role of macrophages in the development of biliary injury in a lipopolysaccharide-aggravated hepatic ischaemia-reperfusion model

J Reiling,^{1,2,3,4} KR Bridle,^{1,2} FG Schaap,^{4,5} L Jaskowski,^{1,2} N Santrampurwala,^{1,2} LJ Britton,^{1,2,6} CM Campbell,⁷ PLM Jansen,⁴ SWM Olde Damink,^{4,5} DHG Crawford,^{1,2} CHC Dejong,^{4,5} J Fawcett^{1,2,3,8}

¹School of Medicine, The University of Queensland, Brisbane, Australia,

²Gallipoli Medical Research Institute, Greenslopes Private Hospital, Newdegate street, Greenslopes, QLD, 4120, Australia,

³PA Research Foundation, Princess Alexandra Hospital, 199 Ipswich road, Woolloongabba, QLD, 4102, Australia,

⁴Department of Surgery, NUTRIM School of Nutrition and Translational Research in Metabolism, Maastricht University, Universiteitssingel 40, 6229 ER Maastricht, the Netherlands,

⁵Department of General, Visceral and Transplantation Surgery, RWTH University Hospital Aachen, Aachen, Germany.

⁶Department of Gastroenterology, Princess Alexandra Hospital, 199 Ipswich Road, Woolloongabba, QLD, 4102, Australia

⁷Envoi Specialist Pathologists, 5/38 Bishop St, Kelvin Grove QLD 4059, Australia

⁸Queensland Liver Transplant Service, Princess Alexandra Hospital, 199 Ipswich Road, Woolloongabba, QLD, 4102, Australia

Corresponding authors:

Janske Reiling, MD, PhD

School of Medicine, The University of Queensland

Lower Lobby Level, Admin Building, Greenslopes Private Hospital

GREENSLOPES Qld 4120, Australia

Ph: +61733460691, Fax: +61733947789 Email: j.reiling@uq.edu.au

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Abstract

Introduction: Endotoxins, in the form of lipopolysaccharides (LPS), are potent inducers of biliary injury. However the mechanism by which injury develops remains unclear. We hypothesized that hepatic macrophages are pivotal in the development of endotoxin-induced biliary injury and that no injury would occur in their absence.

Material and methods: Clodronate liposomes were used to deplete macrophages from the liver. Forty-eight rats were equally divided across six study groups: sham operation (sham), liposome treatment and sham operation (liposomes+sham), 1 mg/kg LPS i.p. (LPS), liposome treatment and LPS administration (liposomes+LPS), hepatic ischaemia-reperfusion injury with LPS administration (IRI+LPS) and liposome treatment followed by IRI+LPS (liposomes+IRI+LPS). Following six hours of reperfusion, blood, bile, and liver tissue was collected for further analysis. Small bile duct injury was assessed, serum liver tests were performed and bile composition was evaluated. The permeability of the blood-biliary barrier (BBB) was assessed using intravenously administered horseradish peroxidase (HRP).

Results: The presence of hepatic macrophages was reduced by 90% in LPS and IRI+LPS groups pre-treated with clodronate liposomes ($P < 0.001$). Severe small bile duct injury was not affected by macrophage depletion, and persisted in the liposomes+IRI+LPS group (50% of animals) and liposomes+LPS group (75% of animals). Likewise, BBB impairment persisted following macrophage depletion. LPS-induced elevation of the chemokine M_{cp}-1 in bile was not affected by macrophage depletion.

Conclusions: Depletion of hepatic macrophages did not prevent development of biliary injury following LPS or LPS-enhanced IRI. Cholangiocyte activation rather than macrophage activation may underlie this injury.

Keywords: Macrophages, Cholangiocytes, Endotoxaemia, Donation after circulatory death, Liver transplantation

1. Introduction

Ischaemic-type biliary stricture (ITBS) formation remains one of the most troublesome complications following liver transplantation using livers donated after circulatory death (DCD) (1, 2). During the DCD retrieval process, ischaemia of intra-abdominal organs such as the gut occurs, and this may result in the release of endotoxins in the portal circulation upon reperfusion (3). We previously showed that endotoxins, in the form of lipopolysaccharides (LPS), are potent inducers of biliary injury (4). Administration of LPS in a hepatic ischaemia-reperfusion injury (IRI) model resulted in a prompt and severe small bile duct injury, which was characterised by degenerative changes of cholangiocytes and ductular proliferation. The injury was associated with the development of the clinical features of cholestasis and increased leakage of macromolecules across the blood-biliary barrier (BBB) (4). In the present study, we further explored the mechanisms by which LPS induced biliary injury in this model by addressing the involvement of hepatic macrophages.

Kupffer cells, the liver-resident macrophages, are activated by binding of LPS to Toll-like receptor-4 (TLR-4) (5). Kupffer cell activation plays a pivotal role in the development of hepatic IRI (6). Upon activation by endogenous damage-associated and/or pathogen-associated molecular pattern (DAMP/PAMP) molecules, Kupffer cells produce reactive oxygen species and pro-inflammatory cytokines, which result in the recruitment of neutrophils and the development of tissue damage (7). Through enhanced Kupffer cell activation, endotoxemia can substantially aggravate IRI, as was observed during organ retrieval and liver transplantation (7-10). Moreover, primary graft non-function following DCD liver transplantation was associated with a higher degree of Kupffer cell activation (11). In animal models, IRI and endotoxin-aggravated IRI was ameliorated by depleting Kupffer cells, as evidenced by reduced hepatocellular damage and decreased recipient mortality (8, 12). It is currently unclear whether hepatic macrophages have a role in the development of endotoxin-induced biliary injury.

The role of cholangiocytes in immune responses is increasingly recognised (13). They express various TLRs, and upon activation they release pro-inflammatory cytokines such as tumor necrosis factor- α (Tnf- α) and interleukin-6 (Il-6) in the bloodstream and biliary compartment (13, 14). Additionally, interferon- γ (IFN γ) and Tnf- α increase the expression of *Tlr4* on cholangiocytes, augmenting their response to LPS *in vitro* (15).

Lastly, cholangiocytes can act as antigen-presenting cells as they express major histocompatibility complex II (MHC II) on their surface (16). Collectively, these data suggest that endotoxin-induced damage of the cholangiocyte might be a direct effect and/or might be mediated via a Kupffer cell response.

The aim of the current study was to explore the role of hepatic macrophages in the development of biliary injury resulting from LPS administration in a hepatic IRI model. We hypothesised that in the absence of macrophages, the BBB would remain intact and biliary injury would be ameliorated.

2. Material and Methods

2.1 Animals and experimental groups

Male Sprague-Dawley rats (250g; Animal Recourses Centre, Perth, Australia) were used for this study. Procedures were in accordance with the Australian Code for the Care and Use of Animals for Scientific Purposes and approved by the Animal Ethics Committee of the University of Queensland. All animal experiments complied with ARRIVE guidelines.

For the purpose of the study, forty-eight animals were equally divided over six study groups: (1) laparotomy without any additional intervention (sham); (2) administration of 1 mg/kg LPS in the peritoneal cavity (LPS); (3) 30 minutes of liver ischaemia and simultaneous administration of LPS in the peritoneal cavity (IRI+LPS); (4) pre-treatment with clodronate liposomes followed by laparotomy (liposomes+sham); (5) pre-treatment with clodronate liposomes followed by administration of LPS in the peritoneal cavity (liposomes+LPS) and (6) pre-treatment with clodronate liposomes followed by 30 minutes of liver ischaemia plus reperfusion and simultaneous administration of LPS in the peritoneal cavity (liposomes+IRI+LPS). Data from animals in groups 1, 2 and 3 (no liposome treatment) was taken from our earlier study (4).

2.2 Macrophage depletion and surgical procedure

Clodronate encapsulated in liposomes (5 mg clodronate per milliliter, www.clodronateliposomes.com, Amsterdam, the Netherlands) was used to deplete hepatic macrophages (17). Animals in groups 4, 5 and 6 were injected with clodronate liposomes (0.5 ml/100 grams body weight) in the tail vein 48 hours prior to surgical intervention. Treatment with control (PBS) liposomes was not conducted as this can result in saturation of the phagocytotic function of macrophages (18).

Midline laparotomy was performed under general anaesthesia with isoflurane and the common bile duct, hepatic artery and portal vein of the left lateral and medial lobes were identified. A vascular clamp (BH030R, BBraun, Bethlehem, PA, USA) was then placed across these structures to induce ischaemia to approximately 70% of the liver (IRI groups). Simultaneously, vehicle (0.9% sterile saline) or 1 mg/kg LPS from *Escherichia coli* serotype 0111:B4 (L3012, Sigma-Aldrich, St. Louis, MO, USA; 1 µg/µl in sterile saline) was administered in the peritoneal cavity. Following 30 minutes

of ischaemia, the clamp was removed and the animals were allowed to recover. The LPS was not rinsed from the abdomen prior to wound closure.

Following six hours of reperfusion, re-laparotomy was performed and a cannula was inserted in the common bile duct to allow for bile collection. Following the establishment of a steady bile flow, three bile samples were collected over 10-minute intervals. Animals were subsequently euthanised by exsanguination, and liver tissue from the left lateral and median lobes (subjected to ischaemia in the IRI groups) was collected for RNA extraction and histological evaluation.

2.3 Immunohistochemical staining

Formaldehyde-fixed paraffin-embedded liver sections of 4 μm thickness were stained with a monoclonal antibody against CD68 and CD163 (ED-1 and ED-2, resp., Serotec, Oxford, UK) to visualise the presence of mature macrophages including Kupffer cells ($\text{CD68}^+ \text{CD163}^+$) as well as their non-phagocytotic precursors ($\text{CD68}^+ \text{CD163}^-$). A monoclonal antibody against Ki67 (#M7248, Dako, Santa Clara, CA, USA) was used to assess proliferation. A TUNEL assay was used to detect DNA fragmentation in apoptotic cells according to the manufacturer's instruction (TUNEL, #S7100, Merck Millipore, Billerica, MA, USA). The average number of CD 163, CD68, Ki67 and TUNEL positive cells per field was counted in five random, non-overlapping fields at 200x magnification using ImageJ image analysis software (19). The number of Ki67 and TUNEL positive biliary epithelial cells per field was expressed as percentage of total biliary epithelial cells.

2.4 Serum and bile analysis

Serum alanine transaminase (ALT), aspartate transaminase (AST), γ -glutamyl transferase (GGT), alkaline phosphatase (ALP), and bilirubin, as well as lactate dehydrogenase (LDH) in bile, were assayed using commercially available kits (Bio Scientific Corporation, Austin, TX, USA). Total bile salt concentration in serum, as well as bile salty and phospholipid concentration in bile were measured in the first collected bile fraction using an enzymatic assay according to the manufacturer's instructions (#80460, Crystal Chem, Inc., Chicago, IL, USA and #433-36201, Wako, Osaka, Japan).

2.5 Blood-biliary-barrier integrity assessment

Horseradish peroxidase (HRP, Peroxidase from horseradish type II, P8250, Sigma-Aldrich, St. Louis, MO, USA) was used to assess the permeability of the BBB *in vivo* as previously described (20). Two hundred microliter of HRP (5000 IU/mL in sterile water) was injected into the inferior vena cava of each animal 30 minutes after cannulation of the common bile duct. Bile was subsequently collected for 10 minutes to assess paracellular transport across tight junctions (21). A commercially available kit (#A22188, Thermo Fisher Scientific, Waltham, MA, USA) was used to determine activity of HRP in bile.

2.6 RNA extraction and real-time polymerase chain reaction

Total RNA was extracted from liver tissue using Trisure (Bioline, Taunton, MA) and cDNA was synthesised using a kit (SensiFAST, Bioline, Taunton, MA, USA). Real-time quantitative polymerase chain reaction (RT-qPCR) was performed on a Viia7 Real-time PCR system (Invitrogen, Carlsbad, CA, USA) using Sybr Green chemistry (Bioline, Taunton, MA, USA). Normalisation was performed using the geometric mean of glyceraldehyde phosphate dehydrogenase, β_2 -microglobulin and basic transcription factor 3 mRNA expression. The primer nucleotide sequences can be found in Supplementary Table 1.

2.7 Western blot

Thirty microgram total liver protein was heat-denatured and separated on a 10% SDS-PAGE gel. Proteins were transferred onto polyvinylidene fluoride (PVDF) membranes (Biorad, Hercules, USA). Blocking was performed in TBS containing 0.1% Tween-20 and 5% skimmed milk powder. Membranes were probed with primary antibodies against Claudin-1 (519000, Invitrogen, Carlsbad, CA, USA) and Gapdh (MAB376, Merck Millipore, Billerica, MA, USA). Secondary detection consisted of HRP-conjugated goat anti-mouse IgG and goat anti-rabbit IgG antibodies (656120, Invitrogen, Carlsbad, CA, USA), respectively. Immunoreactive bands were visualized using a Supersignal West Femto chemiluminescent kit (Thermo Fisher Scientific, Waltham, USA) on an ImageQuant LAS 500 machine (GE Healthcare Life Sciences).

2.8 Histological evaluation

Paraffin-embedded liver sections, stained with hematoxylin and eosin, were used for the semi-quantitative assessment of small bile duct injury. Depending on the size of the ducts (22), a small bile duct injury severity score (SBDISS) was used as previously described by Cheng et al (23). The score was based on bile duct damage (adapted from the Banff criteria for acute rejection) and ductular proliferation, each of which scored from 0 (absent) to 3 (severe). An expert liver pathologist (CC) who was blinded to study group allocation performed the histological scoring. Endotoxins did not induce injury of the large bile ducts following six hours of exposure (data not shown).

2.9 Cytokine array on bile samples

Biliary concentrations of cytokines were measured to gain insight into the local inflammatory milieu surrounding cholangiocytes. Levels of intercellular adhesion molecule-1 (Icam-1), monocyte chemoattractant protein-1 (Mcp-1), tissue inhibitor metalloproteinase-1 (Timp-1), Il-6, interleukin-10 (Il-10) and L-selectin were determined using a multiplex ELISA array (QAR-CYT-2, RayBiotech, Norcross, GA, USA). Diluted samples of bile from the first 10-minutes collection interval were analysed according to the manufacturer's instructions. Fluorescence intensity was determined and data analysis was performed using the Q-analyser (RayBiotech, Norcross, GA, USA).

2.10 Statistical analysis

GraphPad Prism 7 software (GraphPad, San Diego, CA, USA) was used for statistical analysis. Continuous variables are reported as median [interquartile range] and graphed using box plots. A Kruskal-Wallis test was performed to compare all experimental groups. In the case of a significant outcome this was followed by three pre-defined post-hoc comparisons with Dunn's correction for multiple comparisons to evaluate the effect of macrophage depletion: sham versus liposomes+sham, LPS versus liposomes+LPS and IRI+LPS versus liposomes+IRI+LPS. Categorical variables are reported as frequencies (%) and represented in tabular form. A Fisher's exact test was performed to compare categorical data. Eight animals were studied per group unless stated otherwise and a *P*-value <0.05 was considered statistically significant.

The isolated or combined effects of LPS/IRI in our animal model were previously reported (4). No formal statistical evaluation of these effects (in the groups that was not pretreated with liposomes) was made in the current study.

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3. Results

3.1 Effective depletion of mature macrophages from the liver

Effectiveness of clodronate to deplete mature macrophages, including Kupffer cells, from the liver was studied by CD163 immunohistochemistry (Figure 1). In the sham group there were 105 [90–128] CD163⁺ cells per field. In the LPS and IRI+LPS groups this amounted to 157 [143–176] and 145 [130–170] per field. Following liposome pretreatment, the number of positive cells per field decreased in all groups to 17 (16%) ($P = 0.01$), 16 (10%) ($P < 0.001$) and 13 (11%) ($P < 0.001$) positive cells per field, respectively. In addition, the number of CD68 positive cells per field was halved following liposome pre-treatment (Suppl. Figure 1). Thus, clodronate liposomes effectively reduced the numbers of mature macrophage in the liver.

3.2 Macrophage depletion causes elevated liver function tests

Serum ALT activity was not affected by hepatic macrophage depletion (Figure 2A). However AST was significantly increased in the macrophage-depleted groups compared to their non-depleted controls (sham: $P = 0.001$, LPS: $P = 0.03$ and IRI+LPS $P = 0.003$, Figure 2B). LPS-induced hyperbilirubinemia, as noted in our previous study (4), was ameliorated upon depletion of macrophages (Figure 2C). On a transcriptional level, the expression of ATP-binding cassette protein *Mrp2*, which facilitates the biliary secretion of glucuronidated bilirubin, remained unaltered in the macrophage-depleted LPS and IRI+LPS groups (Suppl. Figure 2A). Following macrophage depletion, the previously noted serum elevation of bile salts tended to improve in the LPS group ($P = 0.06$) but remained elevated in the IRI+LPS group (Figure 2D). This occurred despite unaltered expression of Na⁺-taurocholate co-transporting polypeptide (*Ntcp*), the transporter responsible for basolateral uptake of conjugated bile salts, in all groups (Suppl. Figure 2B). Cholestatic injury markers ALP and GGT were both significantly increased following macrophage depletion in the LPS-treated groups (Figure 2E,F).

3.3 Macrophage depletion does not ameliorate small bile duct injury

A small bile duct injury severity score (SBDISS) was calculated. Representative portal tract images are depicted in Figure 3A. None of the animals developed small bile duct injury in the group undergoing sham surgery. In line with our earlier observations, LPS

administration induced severe small bile duct injury (SBDISS>4) in 5 out of 8 (62.5%) animals, and this persisted in 6 out of 8 (75%) animals receiving liposome pre-treatment. In the IRI+LPS group, severe small bile duct injury occurred in 5 out of 8 (62.5%) animals, and this persisted after macrophage depletion (SBDISS>4 in 4 out of 8 animals).

Macrophage depletion did not affect LPS-induced elevation of LDH activity in bile, a biomarker for biliary injury (Figure 3B) (24). Biliary LDH activity tended to be increased in the liposomes+sham group compared to sham controls ($P=0.09$).

3.4 Macrophage depletion prevents LPS-induced hepatocellular apoptosis and induces hepatocyte proliferation.

To further characterize the cellular injury inflicted by LPS, we studied hepatocellular apoptosis and proliferation. Although hepatocytes are typically quiescent, parenchymal injury may trigger cell cycle re-entry to replace lost hepatocytes. Likewise, biliary injury may trigger a proliferative response (ductular reaction).

On average, less than 10% of biliary epithelial cells were positive for Ki67, and this proportion was not affected by macrophage depletion. However, the total number of Ki67⁺ cells per field, primarily representing hepatocytes, was significantly increased in all macrophage-depleted groups (sham: +2.6 fold, LPS: +1.9 fold, IRI+LPS: +1.8 fold, $P < 0.001$ for all groups) (Figure 4A,B).

Macrophage depletion did not induce apoptosis of biliary epithelial cells. Endotoxins however did induce hepatocellular apoptosis in the LPS and IRI+LPS groups ($P < 0.001$) (Figure 4C,D), and this could be prevented by macrophage depletion (LPS: 7 fold reduction of TUNEL⁺ cells, IRI+ LPS: 3 fold reduction of TUNEL⁺ cells, $P < 0.001$).

Necrosis or influx of neutrophils was not observed in any of the groups, and there were no signs of activation of endothelial cells (data not shown).

3.5 Persistent impairment of the BBB following macrophage depletion

In the sham group, macrophage depletion led to a significant increase in the output of intravenously administered HRP in bile suggesting increased BBB permeability (Figure 5A). Elevated biliary HRP activity after LPS administration was not improved by macrophage depletion in either group. We subsequently assessed gene expression levels

of the tight junction protein Claudin-1 (figure 5B). Reduced *Claudin-1* expression in the LPS-only group remained low following liposome pre-treatment, but was significantly increased by macrophage depletion in the IRI+LPS group. Nonetheless, the expression did not exceed that of the untreated sham controls (-1.4 fold Sham, $P=0.19$). LPS treatment or macrophage depletion did not alter Claudin-1 protein levels (Figure 5C,D).

3.6 Macrophage depletion increased bile flow and total bile salt secretion

Bile flow was increased following macrophage depletion, reaching significance in the LPS group. Additionally, bile salt output was increased in both LPS-treated groups following macrophage depletion, while phospholipid output was increased only in the LPS+IRI group (Figure 6A-C). This was accompanied by increased expression of the bile salt export pump (*Bsep*) in the LPS group (Figure 6D). Expression of *Mdr2*, encoding the flippase that facilitates canalicular secretion of phospholipids, remained unaltered in both LPS-treated groups (Figure 6E).

3.7 Biliary cytokine and chemokine levels following macrophage depletion

The biliary concentration of several cytokines and chemokines previously reported to be produced by cholangiocytes were measured to determine the effect of macrophage depletion on the local inflammatory milieu surrounding cholangiocytes (Figure 7 and Supplementary Figure 3). Absolute level of Il-6 was low relative to other factors examined, and was not affected by macrophage depletion in the LPS and IRI+LPS groups (Figure 7A). Clodronate liposome treatment increased biliary Icam-1 protein in the LPS+IRI group to control levels (Figure 7B). Elevation of Mcp-1 in bile in the LPS groups was not affected by macrophage depletion (Figure 7C). LPS-induced elevated Timp-1 levels, as noted in our previous study (4), were further increased upon macrophage depletion in the IRI+LPS group (Figure 7D). Il-10 and L-selectin levels were not affected by LPS administration or LPS-aggravated IRI, but concentrations of these proteins in bile increased upon macrophage depletion (Figure 7E and F).

4. Discussion

In the current study, clodronate liposomes were used to deplete hepatic macrophages from the liver. The development of biliary injury, resulting from exposure to LPS or LPS-aggravated IRI, occurred independently of the presence of hepatic macrophages and impairment of the BBB persisted despite effective depletion of hepatic macrophages. To our knowledge, this is the first study to explore the role of hepatic macrophages in the development of LPS-induced biliary injury.

In this study, cells positive for CD163, a marker of mature macrophages, were largely depleted from the liver following treatment with liposomes. Clodronate liposomes have previously been shown to be highly effective in depleting the mature macrophage population from the liver (25-27). Following intravenous injection, phagocytic cells including Kupffer cells take up the liposomes and apoptosis is induced once toxic intracellular levels of clodronate have been reached (18).

In the current study, serum elevations of AST, ALP and GGT were observed following clodronate liposome treatment. These findings differ from those previously reported. Macrophage depletion from donor livers using gadolinium chloride was found to be beneficial for early graft function and increased survival in animal studies (12). It furthermore reduced hepatic LPS toxicity and endotoxin-aggravated IRI (8, 28). A possible explanation for the increased serum liver enzymes in the present study could be impaired clearance of these enzymes, a role recently attributed to Kupffer cells (25). This was further supported by absence of histological evidence of hepatocellular injury in the liposomes+sham group (data not shown).

LPS-induced hepatocellular apoptosis was ameliorated by macrophage depletion in the current experiment. This may be explained by reduced macrophage production of $\text{Tnf-}\alpha$, an activator of the intrinsic pathway of apoptosis (29). In support of this notion, $\text{Tnf-}\alpha$ gene expression was found to be reduced in the macrophage-depleted groups (data not shown).

An explanation for the increase in the number of proliferating hepatocytes following macrophage depletion could be the release of cell cycle re-entry promoting factors in apoptotic macrophage remnants. In the setting of partial hepatectomy, priming factors released by activated macrophages are known to play an important role in initiating the process of liver regeneration (30).

Despite strong evidence that hepatic macrophages play an important role in the development of hepatic IRI and endotoxin-aggravated hepatic IRI, we did not observe improvement in biliary injury following macrophage depletion. As cholangiocytes can produce cytokines and chemokines in response to LPS exposure, we hypothesised that cholangiocytes could play an important role in the development of biliary injury (13). The LPS-induced elevation of biliary Mcp-1 and Timp-1 protein levels, as observed in our previous study, persisted in the macrophage depleted LPS-groups highlighting the minor role of macrophages in their production. In addition, macrophage depletion had only a small effect on the levels of other cytokines measured in this study. The cellular source of these biliary cytokines is unclear, however cholangiocyte cytokine production may contribute to endotoxin-induced biliary injury. In fact, TLR-4 signalling and subsequent production of cytokines by cholangiocytes has been implicated in the pathophysiology of biliary diseases such as primary sclerosing cholangitis (13). It needs to be noted however that hepatic production of biliary cytokines could not be excluded in this study.

Another explanation for the development of biliary injury upon depletion of hepatic macrophages could be a direct toxic effect of LPS on cholangiocytes. Cells from the reticulo-endothelial system together with hepatocytes are responsible for clearance of agents such as LPS from the portal circulation, and result in secretion of LPS in bile (26, 27, 31). An accumulation of endotoxins has been observed in cholangiocytes of patients with primary biliary cholangitis and primary sclerosing cholangitis (32). Previous studies have shown that endotoxin clearance and secretion into bile can occur in the absence of macrophages, as hepatocytes are able to take up LPS and release it in the biliary space (12, 33). As cholangiocytes would remain exposed to LPS, this could explain why biliary injury developed in the macrophage-depleted groups.

The impairment of the BBB following exposure to LPS persisted upon depletion of macrophages. Macrophages, by clearing endogenous endotoxins, may play a role in maintaining the BBB as increased levels of HRP appeared in bile in the liposomes+sham group. Tight junctions are the main structural components of the BBB, and its permeability can be tested using macromolecules (e.g. HRP) that cross the BBB from the blood stream via the paracellular route (21). Endotoxins as well as individual (pro-inflammatory) cytokines such as Tnf- α , Il-6 and INF γ have previously been found

to impair BBB function (34-36). Even though serum cytokine concentrations are likely reduced following macrophage depletion, the production of cytokines in bile was maintained, which could have affected the BBB function. Alternatively, the increased concentration of HRP in bile observed in the liposomes+sham group could also be the result of reduced phagocytosis of HRP due to the depletion of macrophages. Persistent dysfunction of the BBB could lead to leakage of toxic bile in the surrounding tissues resulting in cellular damage (37). Furthermore, barrier impairment could lead to leakage of bile salts from the canaliculi into the blood stream resulting in increased serum bile salt concentrations as seen in this study.

A limitation of the study is the short duration of the injury, and the fact that cholangiocytes were not separated from other liver cell types for analyses. Cholangiocytes represent a small proportion of the total hepatic cell population, and total hepatic mRNA will in general provide little information on transcriptional changes in cholangiocytes. Furthermore, this study only assessed the short-term effects of LPS administration or IRI, and further work is required to determine if biliary strictures would develop over time. The CD14/TLR4 signalling pathway has previously been linked to the development of ITBS following liver transplantation (38). In addition, in a patient cohort study, immunological factors were associated with stricture formation in the periphery of the liver, affecting smaller bile ducts as was observed in this study (39, 40).

In conclusion, hepatic macrophage depletion did not prevent the development of biliary injury following LPS or LPS-aggravated IRI. Cholangiocytes can produce cytokines in a TLR4- dependent fashion, and results from this study suggest that these cells rather than hepatic macrophages play an important role in LPS-induced biliary injury. Studies using inhibitors of TLR4 or TNF- α signalling, to prevent LPS-induced biliary injury, could provide additional information on the aetiology of LPS-induced biliary injury.

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6. References

1. Laing RW, Scalera I, Isaac J, Mergental H, Mirza DF, Hodson J, et al. Liver transplantation using grafts from donors after circulatory death: A propensity-matched study from a single centre. *Am J Transplant*. 2016.
2. Dubbeld J, Hoekstra H, Farid W, Ringers J, Porte RJ, Metselaar HJ, et al. Similar liver transplantation survival with selected cardiac death donors and brain death donors. *Br J Surg*. 2010;97(5):744-53.
3. Sun Y, Hardaway RM, Williams CH. Comparison of bacterial translocation during traumatic shock and hemorrhagic shock in rats. *International surgery*. 1997;82(2):134-6.
4. Reiling J, Bridle KR, Gijbels M, Schaap FG, Jaskowski L, Santrampurwala N, et al. Low-Dose Lipopolysaccharide Causes Biliary Injury by Blood Biliary Barrier Impairment in a Rat Hepatic Ischemia/Reperfusion Model. *Liver Transpl*. 2017;23(2):194-206.
5. Nakamoto N, Kanai T. Role of toll-like receptors in immune activation and tolerance in the liver. *Frontiers in immunology*. 2014;5:221.
6. Zhai Y, Petrowsky H, Hong JC, Busuttil RW, Kupiec-Weglinski JW. Ischaemia-reperfusion injury in liver transplantation--from bench to bedside. *Nat Rev Gastroenterol Hepatol*. 2013;10(2):79-89.
7. Caraceni P, Pertosa AM, Giannone F, Domenicali M, Grattagliano I, Principe A, et al. Antagonism of the cannabinoid CB-1 receptor protects rat liver against ischaemia-reperfusion injury complicated by endotoxaemia. *Gut*. 2009;58(8):1135-43.
8. Vajdova K, Smrekova R, Kukan M, Jakubovsky J, van Rooijen N, Horecky J, et al. Endotoxin-induced aggravation of preservation-reperfusion injury of rat liver and its modulation. *J Hepatol*. 2000;32(1):112-20.
9. Pillay SP, Wynter C, Lynch S, Wall D, Balderson G, Strong R. Endotoxin levels in donors and recipients during orthotopic liver transplantation. *The Australian and New Zealand journal of surgery*. 1997;67(4):187-91.
10. Pillay SP, Moore TD, Lynch SV, Whiting RF, Balderson GA, Strong RW. Endotoxin levels in adult liver donors. *The Australian and New Zealand journal of surgery*. 1994;64(9):615-7.
11. Monbaliu D, van Pelt J, De Vos R, Greenwood J, Parkkinen J, Crabbe T, et al. Primary graft nonfunction and Kupffer cell activation after liver transplantation from non-heart-beating donors in pigs. *Liver Transpl*. 2007;13(2):239-47.
12. von Frankenberg M, Golling M, Mehrabi A, Nentwich H, Klar E, Kraus TW. Donor pretreatment with gadolinium chloride improves early graft function and survival after porcine liver transplantation. *Transpl Int*. 2003;16(11):806-13.
13. Syal G, Fausther M, Dranoff JA. Advances in cholangiocyte immunobiology. *Am J Physiol Gastrointest Liver Physiol*. 2012;303(10):G1077-86.
14. Harada K, Ohira S, Isse K, Ozaki S, Zen Y, Sato Y, et al. Lipopolysaccharide activates nuclear factor-kappaB through toll-like receptors and related molecules in cultured biliary epithelial cells. *Lab Invest*. 2003;83(11):1657-67.
15. Harada K, Isse K, Nakanuma Y. Interferon gamma accelerates NF-kappaB activation of biliary epithelial cells induced by Toll-like receptor and ligand interaction. *Journal of clinical pathology*. 2006;59(2):184-90.
16. Ayres RC, Neuberger JM, Shaw J, Joplin R, Adams DH. Intercellular adhesion molecule-1 and MHC antigens on human intrahepatic bile duct cells: effect of pro-inflammatory cytokines. *Gut*. 1993;34(9):1245-9.
17. Van Rooijen N, Kors N, vd Ende M, Dijkstra CD. Depletion and repopulation of macrophages in spleen and liver of rat after intravenous treatment with liposome-encapsulated dichloromethylene diphosphonate. *Cell Tissue Res*. 1990;260(2):215-22.
18. Van Rooijen N, Sanders A. Liposome mediated depletion of macrophages: mechanism of action, preparation of liposomes and applications. *Journal of immunological methods*. 1994;174(1-2):83-93.
19. Schneider CA, Rasband WS, Eliceiri KW. NIH Image to ImageJ: 25 years of image analysis. *Nature methods*. 2012;9(7):671-5.
20. Takakuwa Y, Kokai Y, Sasaki K, Chiba H, Tobioka H, Mori M, et al. Bile canalicular barrier function and expression of tight-junctional molecules in rat hepatocytes during common bile duct ligation. *Cell Tissue Res*. 2002;307(2):181-9.
21. Lowe PJ, Kan KS, Barnwell SG, Sharma RK, Coleman R. Transcytosis and paracellular movements of horseradish peroxidase across liver parenchymal tissue from blood to bile. Effects of alpha-naphthylisothiocyanate and colchicine. *Biochem J*. 1985;229(2):529-37.

22. Han Y, Glaser S, Meng F, Francis H, Marzioni M, McDaniel K, et al. Recent advances in the morphological and functional heterogeneity of the biliary epithelium. *Exp Biol Med* (Maywood). 2013;238(5):549-65.
23. Cheng L, Zhao L, Li D, Liu Z, Chen G, Tian F, et al. Role of cholangiocyte bile Acid transporters in large bile duct injury after rat liver transplantation. *Transplantation*. 2010;90(2):127-34.
24. Vajdova K, Smrekova R, Kukan M, Lutterova M, Wsolova L. Bile analysis as a tool for assessing integrity of biliary epithelial cells after cold ischemia--reperfusion of rat livers. *Cryobiology*. 2000;41(2):145-52.
25. Pervin M, Golbar HM, Bondoc A, Izawa T, Kuwamura M, Yamate J. Immunophenotypical characterization and influence on liver homeostasis of depleting and repopulating hepatic macrophages in rats injected with clodronate. *Experimental and toxicologic pathology : official journal of the Gesellschaft fur Toxikologische Pathologie*. 2016;68(2-3):113-24.
26. Freudenberg MA, Galanos C. Bacterial lipopolysaccharides: structure, metabolism and mechanisms of action. *International reviews of immunology*. 1990;6(4):207-21.
27. Hopf U, Ramadori G, Moller B, Galanos C. Hepatocellular clearance function of bacterial lipopolysaccharides and free lipid A in mice with endotoxic shock. *The American journal of emergency medicine*. 1984;2(1):13-9.
28. Iimuro Y, Yamamoto M, Kohno H, Itakura J, Fujii H, Matsumoto Y. Blockade of liver macrophages by gadolinium chloride reduces lethality in endotoxemic rats--analysis of mechanisms of lethality in endotoxemia. *Journal of leukocyte biology*. 1994;55(6):723-8.
29. Baeck C, Tacke F. Balance of inflammatory pathways and interplay of immune cells in the liver during homeostasis and injury. *EXCLI journal*. 2014;13:67-81.
30. Abshagen K, Eipel C, Kalff JC, Menger MD, Vollmar B. Kupffer cells are mandatory for adequate liver regeneration by mediating hyperperfusion via modulation of vasoactive proteins. *Microcirculation*. 2008;15(1):37-47.
31. Mimura Y, Sakisaka S, Harada M, Sata M, Tanikawa K. Role of hepatocytes in direct clearance of lipopolysaccharide in rats. *Gastroenterology*. 1995;109(6):1969-76.
32. Sasatomi K, Noguchi K, Sakisaka S, Sata M, Tanikawa K. Abnormal accumulation of endotoxin in biliary epithelial cells in primary biliary cirrhosis and primary sclerosing cholangitis. *J Hepatol*. 1998;29(3):409-16.
33. Scott MJ, Billiar TR. Beta2-integrin-induced p38 MAPK activation is a key mediator in the CD14/TLR4/MD2-dependent uptake of lipopolysaccharide by hepatocytes. *J Biol Chem*. 2008;283(43):29433-46.
34. Sheth P, Delos Santos N, Seth A, LaRusso NF, Rao RK. Lipopolysaccharide disrupts tight junctions in cholangiocyte monolayers by a c-Src-, TLR4-, and LBP-dependent mechanism. *Am J Physiol Gastrointest Liver Physiol*. 2007;293(1):G308-18.
35. Hanada S, Harada M, Koga H, Kawaguchi T, Taniguchi E, Kumashiro R, et al. Tumor necrosis factor-alpha and interferon-gamma directly impair epithelial barrier function in cultured mouse cholangiocytes. *Liver Int*. 2003;23(1):3-11.
36. Capaldo CT, Nusrat A. Cytokine regulation of tight junctions. *Biochim Biophys Acta*. 2009;1788(4):864-71.
37. Fickert P, Fuchsichler A, Wagner M, Zollner G, Kaser A, Tilg H, et al. Regurgitation of bile acids from leaky bile ducts causes sclerosing cholangitis in Mdr2 (Abcb4) knockout mice. *Gastroenterology*. 2004;127(1):261-74.
38. Friedrich K, Smit M, Brune M, Giese T, Rupp C, Wannhoff A, et al. CD14 is associated with biliary stricture formation. *Hepatology*. 2016.
39. op den Dries S, Buis CI, Adelmeijer J, Van der Jagt EJ, Haagsma EB, Lisman T, et al. The combination of primary sclerosing cholangitis and CCR5-Delta32 in recipients is strongly associated with the development of nonanastomotic biliary strictures after liver transplantation. *Liver Int*. 2011;31(8):1102-9.
40. Op den Dries S, Sutton ME, Lisman T, Porte RJ. Protection of bile ducts in liver transplantation: looking beyond ischemia. *Transplantation*. 2011;92(4):373-9.

7. Figure Legends

- Figure 1** **Successful depletion of mature macrophages following clodronate liposome treatment.** Groups of rats were pre-treated for 48 hours with clodronate liposomes and compared to animals that did not receive liposomes. Pre-treatment was followed by sham operation, administration of lipopolysaccharides (LPS), or a combination of LPS and 30 minutes warm hepatic ischaemia (IRI+LPS). Animals were sacrificed after 6 hours of reperfusion. A) Representative light microscopy images of liver sections (200x magnification) stained for CD163 of animals pretreated with liposomes (bottom row) and those receiving no pretreatment (top row). B) Quantification of CD163⁺ cells per high-powered field. Data is graphed using box plots presenting median, minimum and maximum values. Statistical significance was evaluated using a Kruskal-Wallis test, and -if appropriate- three predefined post-hoc comparisons (sham versus liposomes+ sham, LPS versus liposomes+LPS and IRI+LPS versus liposomes+IRI+LPS). ** $P < 0.01$, **** $P < 0.0001$.
- Figure 2** **Serum liver tests.** Groups of rats were pre-treated for 48 hours with clodronate liposomes and compared to animals that did not receive liposomes. Pre-treatment was followed by sham operation, administration of lipopolysaccharides (LPS), or a combination of LPS and 30 minutes warm hepatic ischaemia (IRI+LPS). Animals were sacrificed after 6 hours of reperfusion and serum activity or level of A) alanine transaminase (ALT, U/L), B) aspartate transaminase (AST, U/L), C) bilirubin (mg/dL), D) total bile salts ($\mu\text{mol/L}$), E) alkaline phosphatase (U/L) and γ -glutamyl transferase (U/L) was determined. * $P < 0.05$, ** $P < 0.01$.
- Figure 3** **Macrophage depletion does not ameliorate LPS-induced biliary injury.** A) Representative light microscopy images of portal tracts (magnification 200x) with arrows indicating bile ducts, PV: portal vein, # hepatic artery. B) Activity of lactate dehydrogenase (LDH) in bile as a biomarker of biliary injury.
- Figure 4:** **Macrophage depletion prevents LPS-induced hepatocellular apoptosis and induces hepatocyte proliferation. Macrophage depletion reduces hepatocellular apoptosis and induces hepatocyte cell cycle re-entry.** Representative light microscopy images of liver sections (200x magnification) stained for Ki67 (A) and TUNEL DNA fragmentation (TUNEL assay) (C) of animals pretreated with liposomes (bottom row) and those receiving no pretreatment (top row). Quantification of Ki67⁺ (B) and TUNEL⁺ cells (D) per high-powered field. Data is graphed using box plots presenting median, minimum and maximum values. Statistical significance was evaluated using a Kruskal-Wallis test, and -if appropriate- three predefined post-hoc comparisons (sham versus liposomes+ sham, LPS versus liposomes+LPS and IRI+LPS versus liposomes+IRI+LPS). **** $P < 0.0001$.
- Figure 5** **Persistent impairment of the blood biliary barrier following depletion of macrophages.** A) Activity of Horseradish peroxidase (HRP) leaking into bile following intravenous injection of 1000 IU HRP. B) Hepatic mRNA expression of *Claudin-1* as assessed using RT-qPCR C) hepatic Claudin-1 protein quantification, D) Claudin-1 Western blot. * $P < 0.05$

Figure 6 **Bile flow, bile composition and gene expression of canalicular transporters.** A) Bile flow ($\mu\text{L}/\text{min}$), B) Total bile salt ($\mu\text{mol}/10\text{ min}$) and C) phospholipid ($\mu\text{g}/10\text{ min}$) output in bile during the first 10-minute collection period D) Hepatic mRNA expression of *Bsep* and E) *Mdr2* determined by RT-qPCR. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Figure 7 **Cytokine and chemokine concentrations in bile.** Biliary concentrations (ng/ml) of Il-6 (A), Icam-1 (B), Mcp-1 (C) and Timp-1 (D), Il-10 (E) and L-selectin (F). * $P < 0.05$, ** $P < 0.01$.

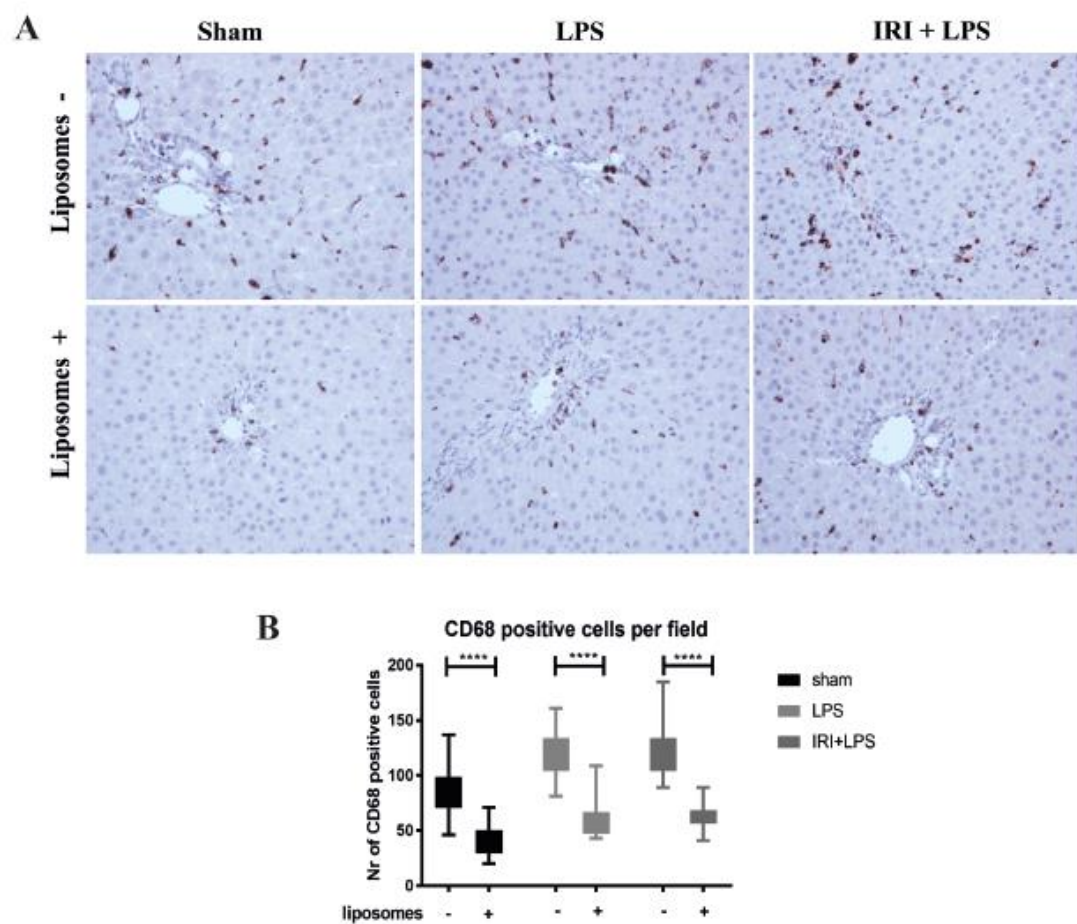


Fig. 1

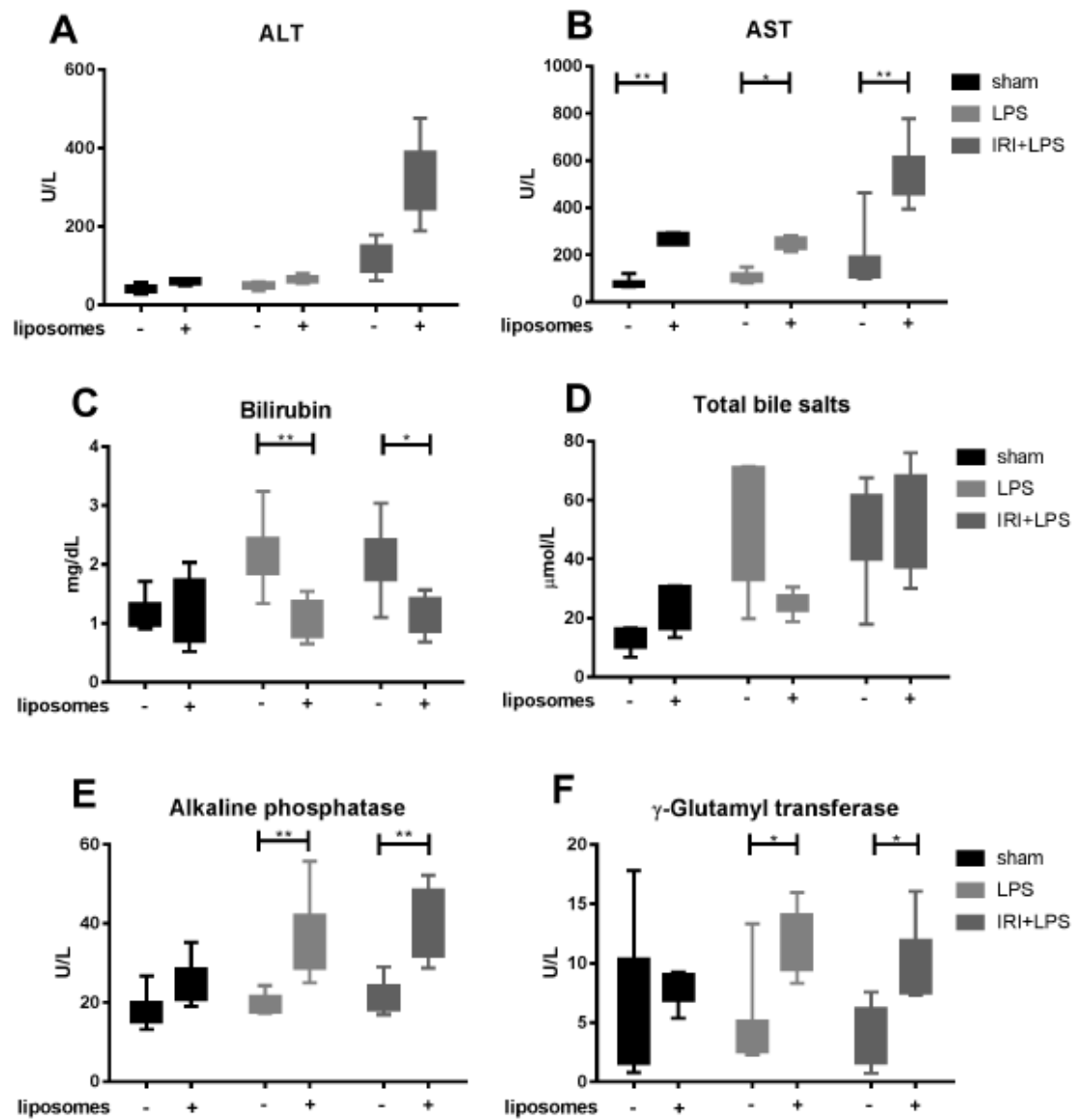


Fig. 2

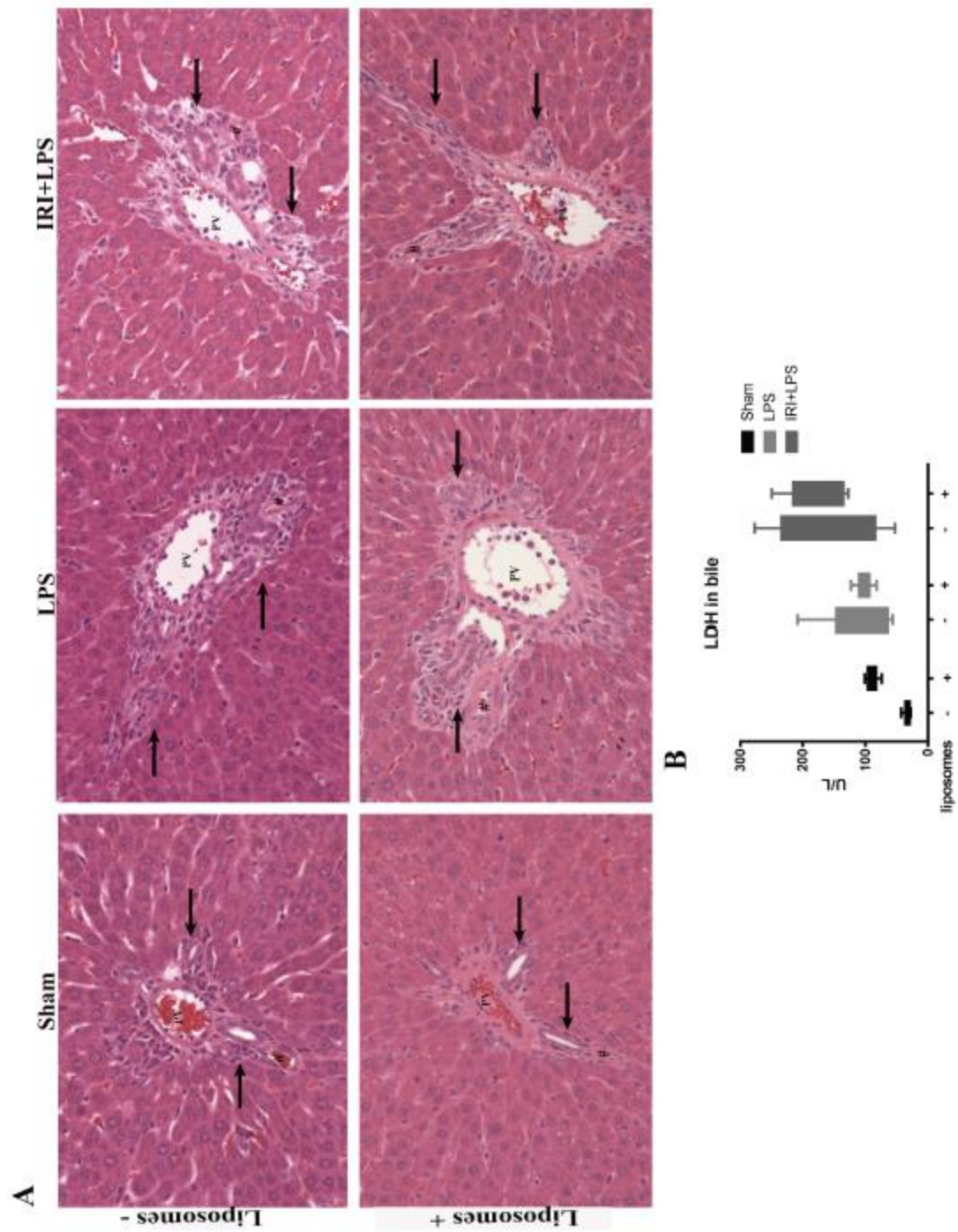


Fig. 3

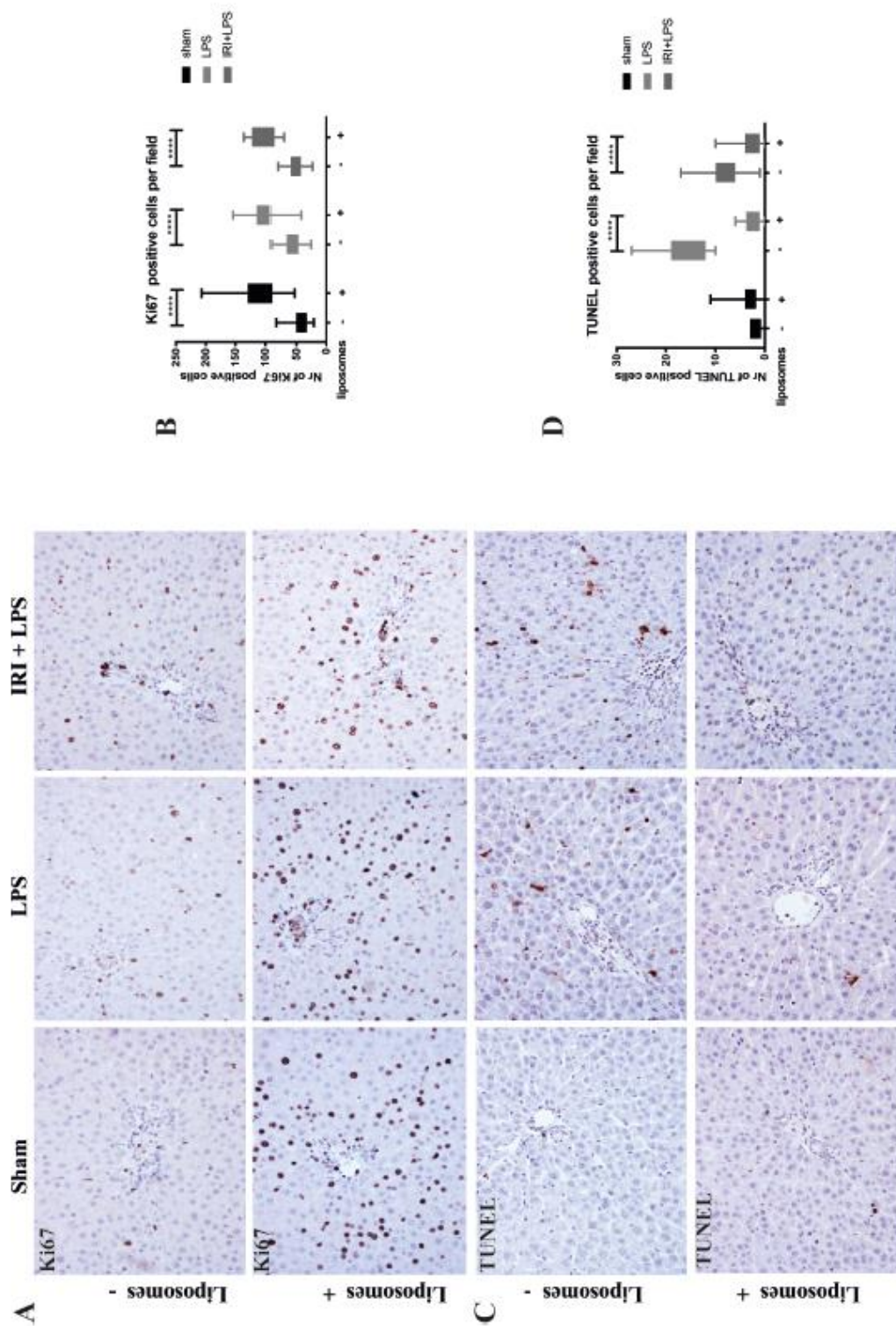


Fig. 4

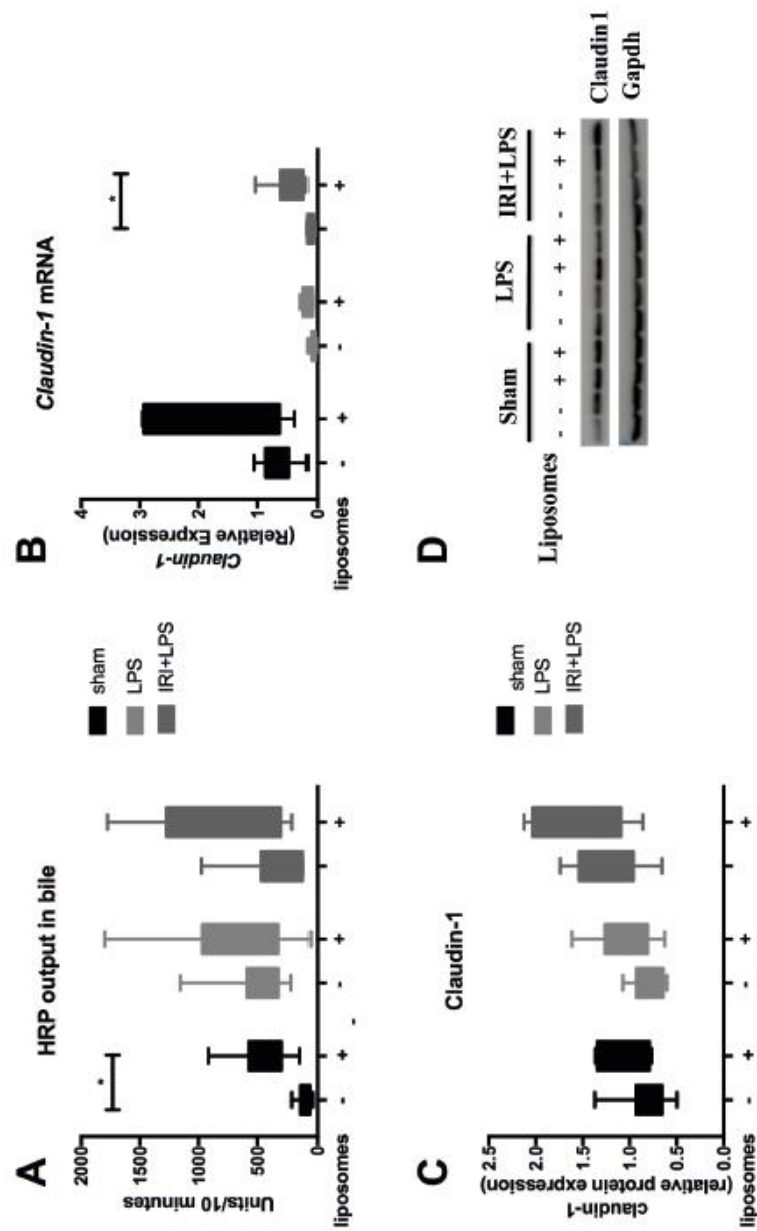


Fig. 5

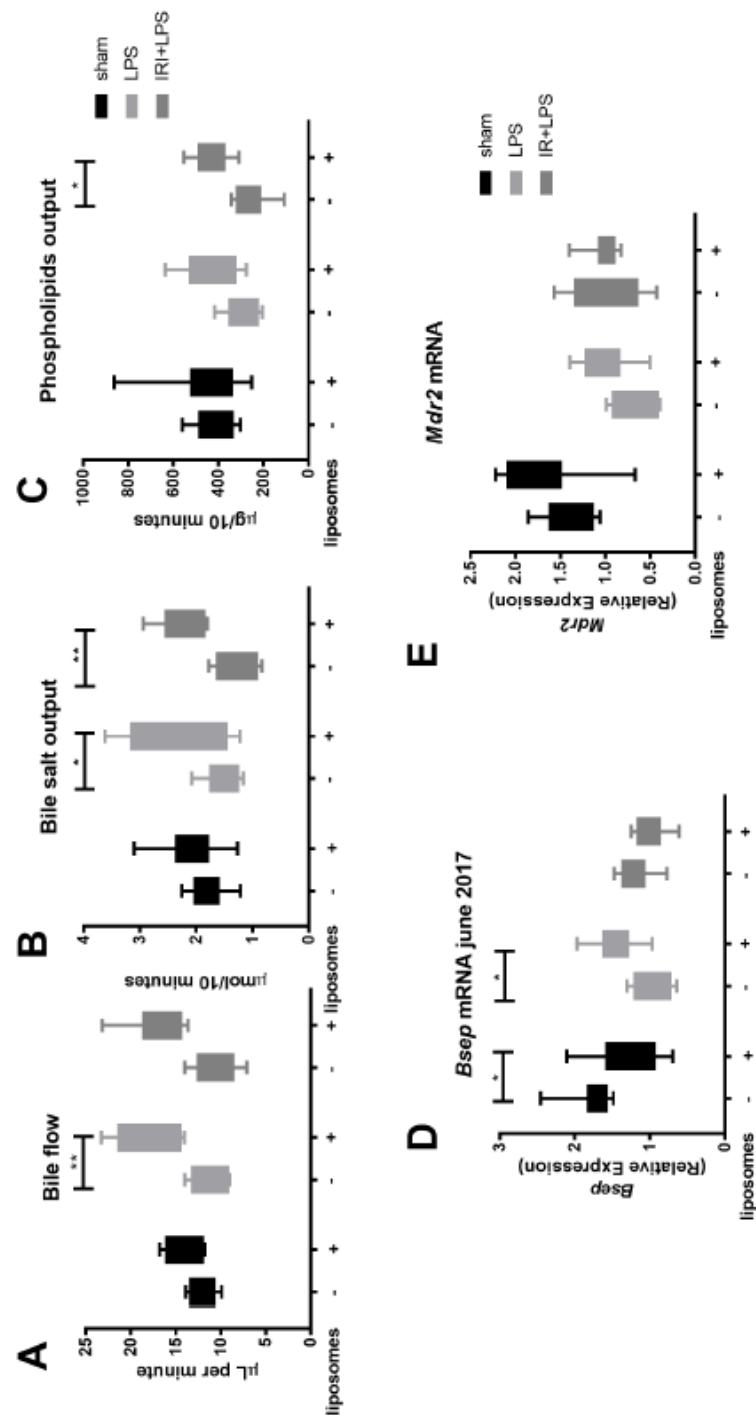


Fig. 6

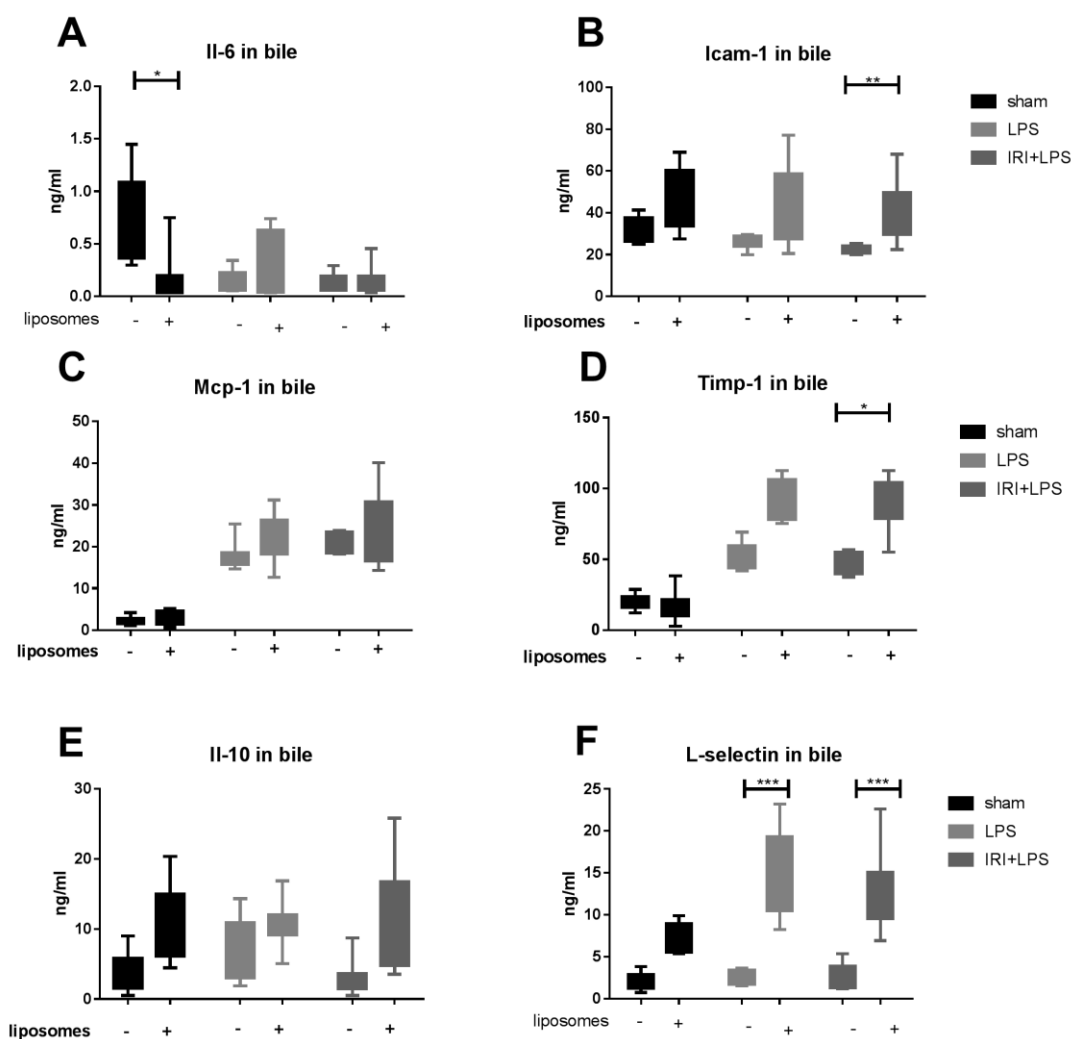


Fig. 7

Highlights

- Endotoxins, in the form of lipopolysaccharides, are potent inducers of biliary injury
- Clodronate liposomes were used to deplete mature macrophages from the liver
- Macrophage depletion did not amend the degree of endotoxin-induced biliary injury
- Cholangiocyte activation rather than macrophage activation may underlie this injury